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The Discovery, Mechanisms, and Evolutionary Impact of Anti-CRISPRs

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Abstract

Bacteria and archaea use CRISPR-Cas adaptive immune systems to defend themselves from infection by bacteriophages (phages). These RNA-guided nucleases are powerful weapons in the fight against foreign DNA, such as phages and plasmids, as well as a revolutionary gene editing tool. Phages are not passive bystanders in their interactions with CRISPR-Cas systems, however; recent discoveries have described phage genes that inhibit CRISPR-Cas function. More than 20 protein families, previously of unknown function, have been ascribed anti-CRISPR function. Here, we discuss how these CRISPR-Cas inhibitors were discovered and their modes of action were elucidated. We also consider the potential impact of anti-CRISPRs on bacterial and phage evolution. Finally, we speculate about the future of this field.

ANTIPHAGE DEFENSE

The genetic diversity of microbes ensures their widespread colonization of the planet. In addition to the challenge of surviving in wide-ranging hostile niches, such as the human body and the depths of the ocean, microbes face a constant onslaught from viruses (1). Bacteriophages (phages)—viruses that infect bacteria—are the most abundant biological entities on the planet, with estimates of $\sim 10^{31}$ particles on Earth (2). Phages are intrinsically specific for the bacterial host that they infect, typically being restricted to a single bacterial species and even a subset of strains within that species. This specificity has enabled careful dissection of the molecular determinants of phage-host interactions in many model bacterial systems, leading to an array of fundamental biological discoveries and groundbreaking biotechnologies.

To defend against phages, diverse antiphage immune mechanisms are found ubiquitously across prokaryotes. These mechanisms can be broadly classified into those that act before phage genome injection and those that manifest after the phage nucleic acid is in the cell. Prior to phage injection, receptors on the cell surface are required for successful phage adsorption. These receptors can be absent, mutated, or masked through specific modifications as an antiphage mechanism (3). Additionally, the poorly understood process of phage genome injection can be inhibited by proteins localizing to the cytoplasmic membrane or periplasm (4–6). Remarkably, many of these antiphage mechanisms are encoded by integrated phages (prophages) and operate through their host as a phage superinfection exclusion mechanism.

Once inside the cell, phages that are entering the lytic cycle hijack host processes to convert the cell into a viral factory. Before phage replication proceeds to completion, the phage nucleic acid (often DNA) may be degraded by bacterial immune systems that target foreign DNA, such as restriction enzymes or CRISPR-Cas nucleases. Many of these antiphage mechanisms have been described in detail in excellent reviews on the subject (see 7, 8). Other intracellular immune systems, such as bacteriophage exclusion in *Bacillus subtilis* (9) and phage-inducible chromosomal island–like elements in *Vibrio cholerae* (10), have recently been discovered, although their mechanisms of action remain obscure.

Despite the numerous powerful systems that bacteria employ to block phage entry and replication, the abundance of phages on the planet shows that these mechanisms have not driven phages to extinction. This can be explained, in part, by a plethora of phage-encoded mechanisms that inhibit these bacterial defenses. Phages can degrade restrictive outer membrane modifications, mutate their tail proteins to utilize alternate receptors, modify their DNA to avoid restriction endonucleases, and encode protein inhibitors of various bacterial processes (11). Here, we discuss the mechanisms by which phages evade CRISPR-Cas function.

CRISPR-CAS SYSTEMS

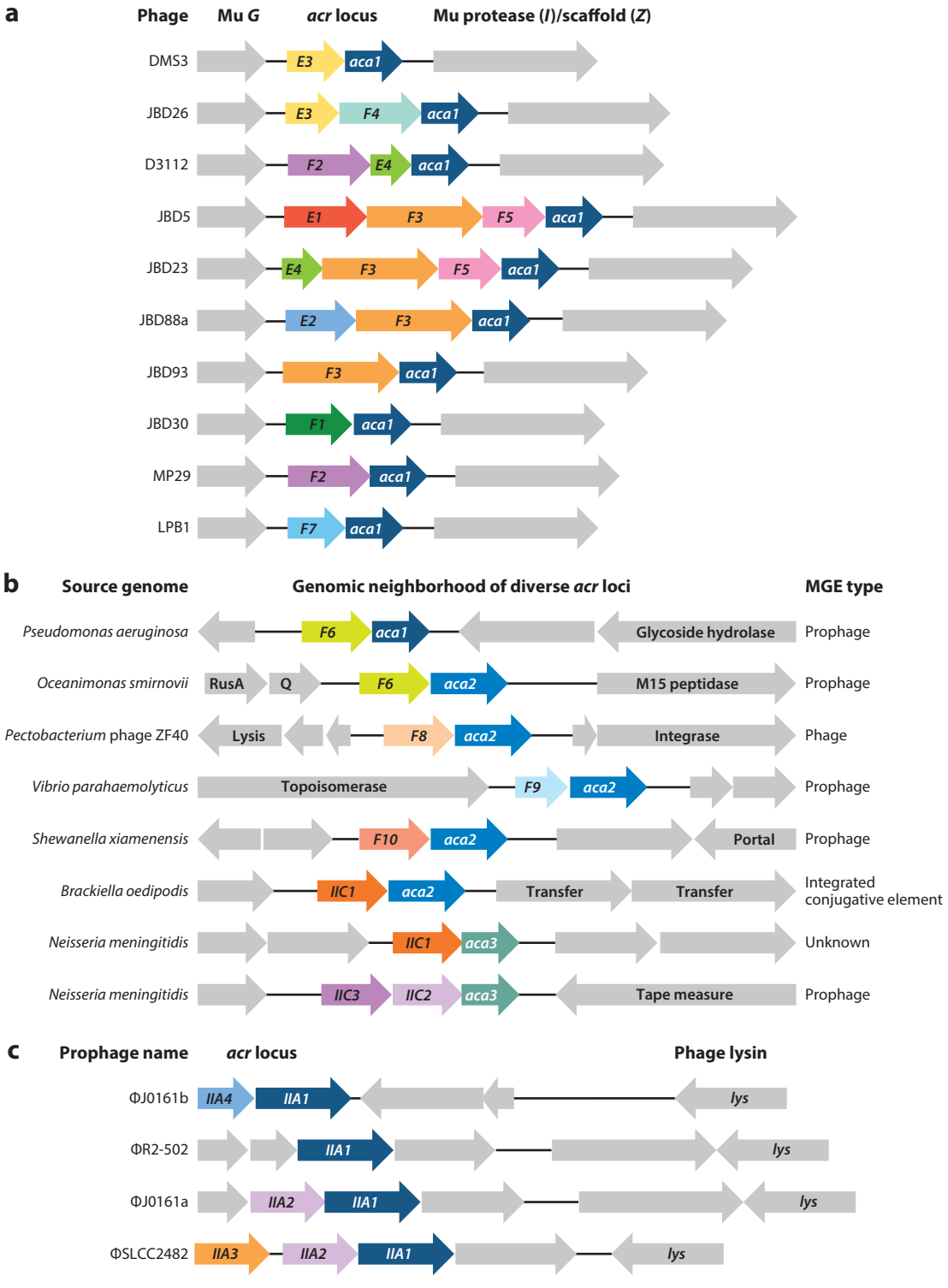
Clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated proteins (Cas) constitute a bacterial adaptive immune system that utilizes RNA-guided nucleases to cleave foreign nucleic acids. By acquiring small fragments of DNA from a foreign element during an initial exposure, the CRISPR array forms a chronological record of past genomic transgressors (12–14). The repetitive elements in the CRISPR array provide semipalindromic functional elements for both the construction of the CRISPR array and the process of interfering with foreign DNA, with spacer elements between the repeats that specify the sequence of the target. In fact, the first hints that CRISPR arrays and *cas* genes might comprise an adaptive immune system against phages was the identification of spacer sequences that are identical to phage genomes (15–17). To function, the CRISPR array is transcribed and processed to generate

mature CRISPR RNAs (crRNAs), which often possess repeat-derived regions at the 5′ and 3′ ends, with the spacer-encoded sequence in the middle (18, 19). This crRNA is assembled with one to six Cas proteins, depending on the type of CRISPR-Cas system, and this complex will surveil the cell (20). Upon recognition of invading complementary nucleic acid, nuclease activity of at least one enzyme is activated and mediates the destruction of that target (21–24). There are currently six known distinct CRISPR-Cas types, which possess completely distinct sets of proteins that enable function. The details of these types and their mechanisms of action have been the subject of excellent reviews (see 25, 26). Here, we focus on the two systems for which anti-CRISPRs have been discovered, type I (Cas3) and type II (Cas9).

Type I CRISPR-Cas systems utilize an RNA-guided protein complex consisting of three to five proteins that process and guide the crRNA to a complementary target and signal for the recruitment of the *trans*-acting nuclease known as Cas3 (18, 27, 28). Type I CRISPR-Cas systems are further categorized into multiple subtypes with distinct RNA-guided protein complexes (I-A through I-F), with all utilizing the Cas3 signature protein for DNA degradation (20). In contrast, type II systems possess a single effector protein, Cas9. Cas9 participates in spacer acquisition, crRNA processing (together with a *trans*-encoded small RNA, termed tracrRNA, and RNase III), target identification, and cleavage (19, 29–32). Type II CRISPR-Cas systems are also broken down into three subtypes (II-A through II-C), possessing distinct Cas9 homologs. Due to the reliance of the type II system on a single protein for function, Cas9 homologs derived from different subtypes and species have been utilized for numerous far-reaching gene editing applications in recent years (33). Both type I and type II CRISPR-Cas systems rely on near-perfect complementarity between the crRNA and a DNA target and on the presence of a subtype-specific protospacer adjacent motif (PAM) (34–36). Point mutations in the PAM or the PAM-proximal region of the protospacer (denoted as the seed) can result in phages or plasmids that escape targeting by the CRISPR-Cas system and proceed to replicate despite a near-perfect or perfect spacer match (37).

ANTI-CRISPR GENES IN *PSEUDOMONAS AERUGINOSA*

In the human pathogen *Pseudomonas aeruginosa*, prophages have been implicated in phenotypes such as toxin production and virulence (38, 39). However, the mechanisms by which *P. aeruginosa* prophages modulate the physiology of their hosts are poorly understood. An effort to discover and characterize novel prophage-mediated phenotypes in this organism led to the serendipitous identification of the first phage-encoded inhibitors of CRISPR-Cas function. A survey of 30 distinct *P. aeruginosa* prophages revealed many examples of superinfection exclusion (40). Surprisingly, examples were observed where a subset of integrated prophages licensed infection by a superinfecting phage, allowing a phage to infect the lysogenized host. This observation was highlighted by a $>10^6$-fold change in the efficiency of plating for phages that did not form plaques on the wild-type, unlysogenized strain but were able to infect and replicate in the lysogenic strain (41). The same phages that could only infect the lysogenized host had been previously shown to be targeted by the natural type I-F CRISPR spacers in the very same wild-type strain (42), leading the authors to speculate that prophages were inactivating CRISPR-Cas function. By comparing the genomes of phages that were sensitive to the action of the CRISPR-Cas system and those that were inactivating it, an anti-CRISPR locus was identified. Many related phages from a single phage family possessed genes in this locus, which were small (i.e., 150–450 bp) and of unknown function. Despite overall synteny and broad conservation of gene sequences throughout the rest of these phages, the anti-CRISPR locus was quite diverse (**Figure 1*a***). When these genes were tested in isolation, five were attributed anti-CRISPR function on the basis of their ability to allow infection by a CRISPR-Cas-targeted phage (41). These genes are now known as *acrF1–5*. In



addition to being encoded by this closely related family of Mu-like phages (i.e., phages that utilize transposition to replicate), homologs of these genes were also identified in conjugative islands and plasmids, suggesting a broad role in enhancing horizontal gene transfer in *Pseudomonas* bacteria (41, 43).

The *P. aeruginosa* type I-F CRISPR-Cas system had been shown to possess noncanonical function prior to anti-CRISPR discovery, via an interaction with a prophage possessing a target sequence with five mismatches. This interaction leads to the inhibition of biofilm formation and swarming motility (44, 45). Furthermore, phages that should have been targeted by the system (i.e., they possessed perfect matches to spacer sequences) were unhindered in their ability to replicate (46). In hindsight, it is now clear that the phages tested with perfect matches possessed *acr* homologs that prevented the detection of CRISPR-Cas activity, and correction of the five mismatches to four or zero mismatches in phage DMS3 (which lacks an *acrF* gene) caused it to be targeted through canonical CRISPR-Cas activity (42). Recent work has revealed that the DMS3 prophage with five mismatches triggers an SOS response as a result of a self-targeting genome cleavage event, which causes death upon the initiation of group behaviors (47). Together, these results highlight that *acrF* genes are important during both lytic and lysogenic growth, to protect a phage with perfect or mismatched protospacer targets. During lysogeny, the constitutive expression of an *acr* gene generates an immunocompromised host, which is now sensitive to other phages that the CRISPR-Cas system would have previously blocked. Although this seems maladaptive for the prophage and lysogen, the inhibition of CRISPR-Cas function is an obligate part of lysogenic survival, as the genome cleavage that would result from self-targeting of the prophage would be lethal in the absence of an anti-CRISPR.

In addition to the type I-F system, type I-E systems were also identified in many *P. aeruginosa* genomes (46). Whereas type I-F function was serendipitously identified in a widely used lab strain (PA14), finding a strain with a functional type I-E system required active searching among sequenced strains possessing this system. Strains with active systems were identified by designing plasmids possessing protospacers and assessing transformation efficiencies. Ultimately, two *P. aeruginosa* strains with functional type I-E systems were found; subsequently, using one of those strains, four distinct type I-E anti-CRISPR genes (*acrE1–4*) were identified (48). The *acrE* genes were found as genomic neighbors to the *acrF* genes in the same family of Mu-like prophages. In many cases, individual phages were identified that had both an *acrF* and an *acrE* gene. A summary of all anti-CRISPR proteins along with their phages of origin and accession numbers is given in **Table 1**.

Figure 1

Anti-CRISPR (*acr*) locus organization. Stereotypical organizations of *acr* loci encoded by phages and mobile genetic elements (MGEs) are shown. Unique *acr* genes are named and shown in color, whereas non-*acr* genes are shown in gray and are annotated with predicted functions when possible. (*a*) *Pseudomonas aeruginosa* Mu-like phage *acr* locus. The *acr* genes are all integrated at the same locus between two highly conserved structural genes (*gray*) that are homologous to Mu phage gene *G* and Mu phage protease (*I*)/scaffold (*Z*) genes. Many loci encode both type I-E (AcrE1–4) and I-F (AcrF1–5) Acr proteins, all adjacent to the conserved anti-CRISPR-associated gene 1 (*aca1*). A representative phage is indicated for each unique locus architecture. Panel adapted from Reference 48. (*b*) *acr* loci in diverse *Proteobacteria* are shown. These *acr* loci do not share a common "genomic neighborhood," but all are anchored by HTH-encoding anti-CRISPR-associated genes (*aca1–3*). Representatives of each *acr-aca* association are shown in the indicated species. Panel adapted from Reference 49. (*c*) Listeriophage *acrIIA* locus. The listeriophage locus is near the left end of the integrated prophage genome and a highly conserved endolysin gene (*lys*). All listeriophage *acr* loci are anchored by the HTH-encoding gene *acrIIA1*. Panel adapted from Reference 51.

Table 1 Sources and accession numbers for all characterized Acr and Aca proteins

Name	Source	Accession number
AcrF1	*Pseudomonas aeruginosa* phage JBD30 (gp35)	YP_007392342.1
AcrF2	*Pseudomonas aeruginosa* phage MP29 (gp29)	YP_002332454.1
AcrF3	*Pseudomonas aeruginosa* phage JBD88a (gp33)	YP_007392440.1
AcrF4	*Pseudomonas aeruginosa* phage JBD26 (gp37)	WP_016068584.1
AcrF5	*Pseudomonas aeruginosa* phage JBD5 (gp36)	YP_007392740.1
AcrF6	*Pseudomonas aeruginosa* prophage	WP_043884810.1
AcrF7	*Pseudomonas aeruginosa* phage LPB1 (gp29)	YP_009146150.1
AcrF8	*Pectobacterium atrosepticum* phage ZF40 (gp31)	YP_007006940.1
AcrF9	*Vibrio parahaemolyticus* prophage	WP_031500045
AcrF10	*Shewanella xiamenensis* prophage	WP_037415910.1
AcrE1	*Pseudomonas aeruginosa* phage JBD5 (gp34)	YP_007392738.1
AcrE2	*Pseudomonas aeruginosa* phage JBD88a (gp32)	YP_007392439.1
AcrE3	*Pseudomonas aeruginosa* phage DMS3 (gp30)	YP_950454.1
AcrE4	*Pseudomonas aeruginosa* phage D3112 (gp31)	NP_938238.1
AcrIIA1	*Listeria monocytogenes* prophage J0161a	WP_003722518.1
AcrIIA2	*Listeria monocytogenes* prophage J0161a	WP_003722517.1
AcrIIA3	*Listeria monocytogenes* prophage SLCC2482	WP_014930691.1
AcrIIA4	*Listeria monocytogenes* prophage J0161b	WP_003723290.1
AcrIIC1	*Neisseria meningitidis* MGE	WP_049360089.1
AcrIIC2	*Neisseria meningitidis* prophage	WP_042743678.1
AcrIIC3	*Neisseria meningitidis* prophage	WP_042743676.1
Aca1	*Pseudomonas aeruginosa* phage JBD30 (gp36)	YP_007392343
Aca2	*Oceanimonas smirnovii* prophage	WP_019933869.1
Aca3	*Neisseria meningitidis* prophage	WP_049360086.1

THE DISCOVERY OF ANTI-CRISPRS IN DIVERSE BACTERIAL SPECIES

Anti-CRISPR loci in the *P. aeruginosa* Mu-like phages possess a stereotypical genomic architecture (**Figure 1*a***), with one to three *acrE* or *acrF* genes followed by a highly conserved gene that is referred to as anti-CRISPR-associated gene 1 (*aca1*). Whereas the anti-CRISPR genes possess no significant sequence identity, *aca1* homologs in this family of phages encode proteins with 95% sequence identity and occur only in phages that possess *acr* genes. Homologs of *acrE* and *acrF* genes have been found in diverse mobile elements within *Pseudomonas* species, but homology searches did not identify any hits outside of this genus, making it difficult to predict whether anti-CRISPRs are widespread. The conservation of *aca1*, however, provided a robust bioinformatics tool to identify novel *acr* genes both within and outside of *Pseudomonas* (**Figure 1*b***). When this conserved gene was used as a query, two new *acrF* genes (*acrF6* and *acrF7*) were discovered in *Pseudomonas* mobile elements. Excitingly, *acrF6* homologs were discovered in diverse gammaproteobacteria, and some of them proved to be active against the *P. aeruginosa* type I-F CRISPR-Cas system, representing the first anti-CRISPRs found outside of the *Pseudomonas* genus. Also important, one *acrF6* homolog was found next to a gene encoding an HTH motif–containing protein that was distinct from that encoded by *aca1*; this gene was named *aca2*. Homologs of *aca2* then led to the discovery of *acrF8–10* in diverse organisms, as well as many other candidates that did not possess anti-CRISPR

activity in *P. aeruginosa*. These anti-CRISPR genes were identified broadly across the phylum *Proteobacteria* (49). Notably, some homologs of each new *acrF* gene discovered in this manner displayed a broad host range, inactivating the type I-F systems of *P. aeruginosa* and *Pectobacterium atrosepticum*. The Cas proteins of the *Pectobacterium* system have 40% to 60% sequence identity with their *P. aeruginosa* orthologs. This broad host range was a feature of only *acrF1* and *acrF2* from the original group. Furthermore, this study yielded the first dual-specificity anti-CRISPR protein (encoded by $acrF6_{Pae}$), which could also inactivate the type I-E CRISPR-Cas system (49). This work demonstrated the power of utilizing a guilt-by-association bioinformatics approach to discover small, novel genes of unknown function next to *aca1* and *aca2* homologs as a method for the discovery of new anti-CRISPRs.

THE DISCOVERY OF ANTI-CRISPRS INHIBITING CAS9

Performing further BLAST searches with Aca2 led to a putative Acr encoded in a strain of *Brackiella oedipodis* (50). This strain did not possess a type I CRISPR-Cas system, but did contain a type II-C system, leading to the hypothesis that the putative Acr might be a Cas9 inhibitor. A homolog of the putative Acr was found in *Neisseria meningitidis*, which also possesses a type II-C system (**Figure 1*b***). Subsequent experiments showed that these proteins did inhibit the *N. meningitidis* Cas9 system in its natural context, proving the existence of anti-CRISPRs against a Cas9-based system. Further bioinformatics investigation uncovered two more families of anti-CRISPRs functioning to inhibit *N. meningitidis* Cas9. Excitingly, these anti-CRISPRs were also found to function in human cells to inhibit genome editing mediated by *N. meningitidis* Cas9. These studies were important in showing that Acr proteins are not limited to type I CRISPR-Cas systems.

When a strain acquires foreign DNA bearing an *acr* gene, one outcome is that it may now possess a target of the CRISPR-Cas system within its genome. For example, a temperate phage with a targeted protospacer and PAM can avoid CRISPR targeting by deploying an Acr protein, allowing integration and stable lysogeny due to continued production of the Acr protein. This results in a situation described as self-targeting. The continued expression of an Acr protein is now an essential process in this cell, as loss of Acr expression will result in lethal genomic cleavage. This premise was utilized as a bioinformatics strategy to identify strains that possessed the first Acr proteins found to be encoded by a gram-positive microbe (51). Four distinct inhibitors of the type II-A CRISPR-Cas system in *Listeria monocytogenes* were identified (*acrIIA1–4*), guided by examples of genomic self-targeting (**Figure 1*c***). Most *acr* genes in this system were encoded by prophages in *L. monocytogenes* genomes; some *acr* homologs were found in distantly related phages and plasmids of *L. monocytogenes* and other members of the phylum *Firmicutes*. Two of the proteins encoded by these newly discovered *acr* genes (AcrIIA2 and AcrIIA4) were able to block function of the widely used *Streptococcus pyogenes* Cas9 both in an *Escherichia coli* test system and in a genome editing assay in human cells. *L. monocytogenes* and *S. pyogenes* Cas9 are 53% identical, showing that AcrIIA proteins can also function against distinctive systems.

The type II-A and type II-C Acr proteins represent important new additions to the Cas9 engineering toolkit, derived from the phage-bacterium arms race. Much work remains to be done to understand how widespread these proteins are, how many distinct proteins exist that perform this task, and what the evolutionary implications are for their presence.

ANTI-CRISPRS ARE WIDESPREAD

Whereas ~50% of bacteria possess CRISPR-Cas systems, it remains an open question whether any given system is active and able to respond to foreign DNA invasion (52). Although it is impossible

to experimentally interrogate every microbe possessing a type I-F system, for example, one can use bioinformatics to predict whether a given system may be capable of being inactivated by known *acr* genes. In the type I-F CRISPR-Cas system, for which ten *acrF* sequences are available, it appears that nearly every known version of the system is found within a genus where isolates also carry a known *acr* gene or a closely related ortholog (49). This suggests widespread inactivation of type I-F CRISPR-Cas systems across their entire distribution and provides a hypothesis going forward: that every CRISPR-Cas system may possess a similar and concomitant abundance of *acr* genes throughout its distribution.

The *acrIIC* genes were also found beyond the organism in which they were discovered (*N. meningitidis*), suggesting the potential for widespread type II-C CRISPR-Cas inactivation as well (50). The coverage was not as striking as in the case of the *acrF* genes, however, suggesting that there are likely more *acrIIC* genes to discover. The *acrIIA* genes told a slightly different story: Whereas homologs of *acrIIA2–4* were found only in *Listeria* and *Streptococcus* prophages and plasmids, homologs of *acrIIA1* were found broadly distributed across the phylum *Firmicutes*. This distribution included many species encoding type II-A CRISPR-Cas systems, suggesting widespread inactivation of Cas9 in these organisms. As the discovery of new *acr* genes continues, it will be exciting to track where their homologs are found to determine what percentage of CRISPR-Cas systems are likely "inhibitable" and what this will mean for bacteria and phages on an evolutionary timescale.

ANTI-CRISPR GENE ORGANIZATION

To defend against commonly encountered type I-E and type I-F CRISPR-Cas systems, many *P. aeruginosa* phages maintain an *acrE* gene alongside an *acrF* gene in their anti-CRISPR loci. The most interesting example of phage response to dual I-E and I-F CRISPR-Cas targeting is the evolution of the *P. aeruginosa* phage allele of *acrF6* (*acrF6*$_{Pae}$), which encodes a single protein possessing dual I-E and I-F inhibitory activity. This dual activity is unique to the *P. aeruginosa* phage allele, as *acrF6* homologs from five other diverse bacteria did not inhibit the type I-E system of *P. aeruginosa*. Interestingly, this anti-I-E activity could be abolished by a C-terminal truncation of the final two residues of the AcrF6$_{Pae}$ protein, while leaving the anti-I-F activity of the protein unaffected. In contrast to the pervasive co-occurrence of heterotypic Acr proteins in *P. aeruginosa* phages, examples of two *acrF* or *acrE* genes appearing together in the same genome are much rarer. The singular locus architecture with two *acrF* genes is the co-occurrence of *acrF3* and *acrF5*. Interestingly, *acrF3* often occurs in the absence of *acrF5*, but *acrF5* is never found without *acrF3*. The functional significance of this unique genetic interaction is unknown. In contrast to I-F and I-E inhibitors, multiple II-A inhibitors are often encoded together in the same anti-CRISPR locus. An estimated 75% of *acrIIA* loci encode more than one AcrIIA protein, whereas only approximately 7% of *acrF* loci have both *acrF3* and *acrF5*, and there are no examples of *acrE* genes occurring in tandem. Dominating the *acrIIA* landscape is *acrIIA1*, which pervasively co-occurs with *acrIIA2–4*, demonstrating a potential multipronged attack on the *L. monocytogenes* CRISPR-Cas system.

MECHANISMS OF ANTI-CRISPR PROTEIN FUNCTION

A notable feature of each family of Acr proteins is their lack of sequence similarity to any proteins of known function. Furthermore, besides being small (~50–150 amino acids), they share no common features among them. For these reasons, no insight into the mechanisms of Acr function could be gained until experimental studies on individual Acr proteins were undertaken. The first such study provided an in vitro mechanistic characterization of AcrF1, AcrF2, and AcrF3, each of which was

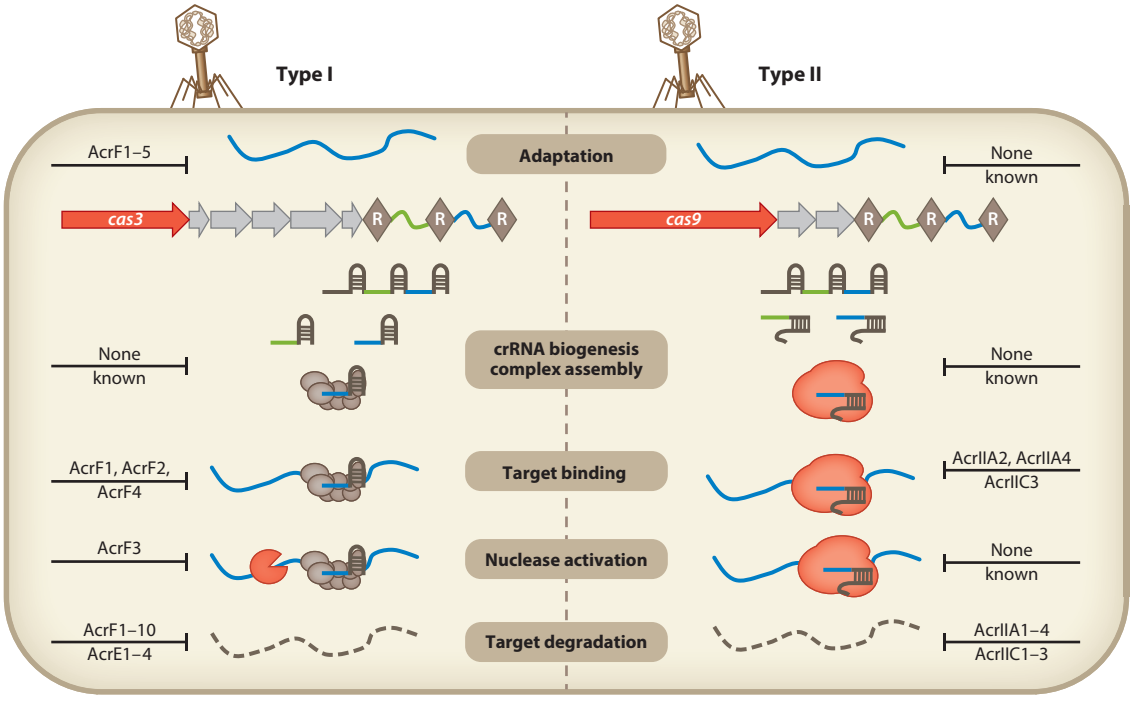


Figure 2

Characterized and predicted mechanisms for anti-CRISPR protein function. CRISPR-Cas immune function is broken down into five distinct processes, shown in brown boxes. Acr proteins that inhibit these processes are shown for both type I and type II CRISPR-Cas systems. All characterized type I-F Acr proteins (AcrF1–5) have been demonstrated to inhibit both adaptation and immunity by preventing either foreign DNA recognition (AcrF1, AcrF2, and AcrF4) or Cas3 nuclease recruitment (AcrF3). AcrIIA2, AcrIIA4, and AcrIIC3 prevent DNA target binding by Cas9. All anti-CRISPRs are defined by their ability to ultimately prevent foreign DNA destruction, though the mechanisms by which most of them accomplish this task are still unknown. Abbreviations: crRNA, CRISPR RNA; R, repeat.

found to function through a different mechanism (53). AcrF1 and AcrF2 both bound to the type I-F CRISPR-Cas (Csy) complex, but they did this by binding to different subunits of the complex (**Figure 2**). AcrF1 bound with a stoichiometry of two or three to the Cas7f (Csy3) subunit, which is present in six copies in the Csy complex. By contrast, AcrF2 bound to the Csy complex with a stoichiometry of one and interacted with the Cas8f:Cas5f (Csy1:Csy2) heterodimer. Both AcrF1 and AcrF2 inhibited the DNA-binding activity of the Csy complex. However, AcrF2 directly competed with DNA for a site on the Csy complex, whereas AcrF1 interacted with a site removed from the DNA interaction site. Interestingly, AcrF1 could still associate with the DNA-bound Csy complex if the DNA was added first. AcrF3 directly bound to the Cas3 helicase-nuclease protein and prevented its recruitment to the Csy-DNA complex. AcrF4 bound to the Csy complex, but specific details of this interaction were not obtained (53).

To gain structural resolution of AcrF3 interacting with Cas3, cocrystal (54) and cryoelectron microscopy (55) structures were recently published. These structures revealed an AcrF3 dimer, in which each monomer makes multiple asymmetric contacts with many residues and domains of Cas3. This effectively covers an entire face of the Cas3 protein, approximately 2,500 $Å^2$ in surface area (54). In contrast to these results with AcrF3, a nuclear magnetic resonance solution structure of AcrF1 coupled with extensive mutagenesis revealed a small patch of the protein was required

for function (56). A single tyrosine-to-alanine mutation at position 6 of AcrF1 was sufficient to inactivate anti-CRISPR function in vivo and in vitro. These two Acr protein structures highlight the structural and mechanistic diversity of these inhibitor proteins.

OTHER POSSIBLE ANTI-CRISPR MECHANISMS

In contrast to newly discovered CRISPR/anti-CRISPR antagonism, other forms of bacterial immunity/counterimmunity are better studied and their evolution better understood (57). Biomimicry is employed as a mechanism to inhibit immune activity across diverse systems. This leads us to hypothesize that Acr proteins could have evolved by mechanisms of biomimicry and bacterial gene hijack.

Nucleic Acid Mimics

Restriction enzymes comprise the bacterial innate immune system and have been studied for many years (58). Diverse inhibitors of restriction enzyme immunity have been discovered, many of which ultimately function by shielding the phage DNA from enzymatic attack using base modification (59). However, other inhibitors work by mimicking phage DNA and tightly sequestering restriction enzymes (60). The T7 *ocr* gene, an immediate-early gene that T7 uses to inhibit restriction activity in its *E. coli* host, encodes a protein that is highly acidic and structurally resembles 24 bp of bent B-form DNA (61). Similarly, the protein encoded by the *ardA* gene, a widely distributed inhibitor of type I restriction systems, functions as a homodimer that mimics a 42-bp stretch of B-form DNA (62). Viral biomimicry of DNA is also seen in eukaryotic systems, where a virally encoded DNA mimic binds histones and is hypothesized to disrupt nucleosome assembly and prevent repair of DNA breaks (63).

Like restriction enzymes, CRISPR-Cas systems bind DNA and, in principle, should be susceptible to inhibition by DNA mimics. DNA-binding activities that are independent of the sequence of the spacer-derived crRNA (i.e., the PAM site) ascribed to both type I and type II CRISPR-Cas systems could provide a weakness for anti-CRISPR DNA mimics to exploit. Although inhibitors of RNA-binding CRISPR-Cas systems (64) have not yet been reported, RNA biomimicry could similarly function as an anti-CRISPR strategy. Furthermore, we hypothesize that virally encoded small RNAs could mimic crRNAs and interfere with CRISPR-Cas activity. Specifically, crRNA mimics could function by outcompeting bona fide crRNAs for Cas proteins during CRISPR-complex assembly, or by directly displacing crRNAs in preloaded complexes. Interestingly, some *Clostridium* phages carry CRISPR arrays, the biological function of which is unknown (65).

Cas Proteins as Proto-Anti-CRISPRs

Horizontal gene transfer between phages and host bacterial species is pervasive, and CRISPR-Cas elements have been found in phage genomes previously. In a striking example of horizontal Cas gene transfer, *V. cholerae* phages acquired a type I-F CRISPR-Cas system that they deploy to inhibit a novel DNA-based antiphage immune system (10). Because Cas proteins interact in complex with each other, an Acr protein that mimics a Cas protein or Cas protein motifs could compete with or disrupt these bona fide Cas-Cas interactions. Despite these predictions, the structures of *P. aeruginosa* AcrF1 (56) and AcrF3 (54, 55) bear no obvious resemblances or topological similarities to any of the *P. aeruginosa* Cas proteins for which there are structures: Cas1, Cas2/3, or Cas6 (18, 54, 66). Further structural characterization of Cas:Acr protein interactions is urgently needed,

especially for the multiprotein complexes utilized in type I systems as the structural intricacies of this complex may be absent without the interaction partners and crRNA present. This information will help illuminate the currently obscure evolutionary history of Acr proteins.

ANTI-CRISPRS AS MODIFIERS OF CRISPR-CAS FUNCTION

The nuances of AcrF function go far beyond simple inhibition of interference, as these proteins have the ability to enable or disable new functions that were not initially predicted. For example, the inhibition of Cas3 recruitment mediated by AcrF3 converted the CRISPR-Cas system into a sequence-specific transcriptional repressor (CRISPR interference, or CRISPRi) when the system was targeted to a promoter region. This repression presumably occurred because the crRNA-guided complex could bind DNA and block RNA polymerase recruitment, but DNA cleavage did not occur (53). Type I CRISPRi had previously been demonstrated in the type I-E system via the deletion of the Cas3 nuclease (67, 68) and in the type II system by catalytic inactivation of Cas9 (69, 70). This demonstrates the ability of an Acr protein to leave CRISPR-Cas function partially intact and may therefore enable new functionalities, such as so-called natural CRISPRi. Additionally, the discovery of priming acquisition (a mechanism of spacer acquisition that requires all components of the CRISPR-Cas system) connected the spacer acquisition and interference pathways, which were previously thought to be separate (14). With this connection, it became clear that the binding of AcrF proteins to the interference factors in the type I-F system (Csy complex, Cas3) also functions to block new spacer acquisition (71).

The recent discovery of Cas9 inhibitors, AcrIIA and AcrIIC, that were able to interfere with Cas9 gene editing activity in human cells (50, 51) suggests that these also directly bind to Cas9. Indeed, AcrIIC1, AcrIIC2, and AcrIIC3 all form direct physical interactions with type II-C Cas9 from *N. meningitidis* but not type II-A Cas9 from *S. pyogenes* (50). These data show the utility for Acr proteins to function in heterologous hosts with potential benefits such as providing an off switch for gene editing applications and Cas9-based CRISPRi applications.

Direct interactions with Cas proteins present a logical solution for phages to inhibit CRISPR-Cas-based immunity, but we envision many distinct strategies to achieve this end result. For example, base modifications have been previously shown to block type II (72) and type I (73) immunity, although the type II results seem to depend on the guide RNA design (74). Certain mechanisms of phage injection and replication may also be recalcitrant to CRISPR targeting, as was recently shown for phage T5, which injects its genome gradually such that only ~10% of the DNA is a substrate for effective CRISPR-Cas immunity (73). Additionally, Acr proteins that conduct enzymatic inactivation or destruction of Cas proteins or the crRNA, or transcriptional repression of any component of the CRISPR-Cas system, might sufficiently shift the balance in favor of the phage during infection.

ANTI-CRISPR GENES ARE PHAGE ACCESSORY GENES

Phage genomes are highly mosaic, possessing distinct functional modules with unique evolutionary histories (1, 75, 76). Individual modules are assorted into phage genomes through diverse mechanisms of DNA recombination and/or ligation, and high-fitness combinations are selected for, whereas low-fitness assemblages are purged from the phage population. The frequency with which modules are moved in or out of genomes of related phages creates a conservation pattern that allows for the designation of core and accessory genes across a population of related phages. The core genome contains genes that are essential for lytic or lysogenic replication under all

conditions and genetic backgrounds, such as genes encoding the phage virion components, lysis proteins, or repressor proteins (77, 78). Whereas core genes are broadly conserved among groups of related phages, accessory genes are often conserved in only subsets of phages and may also be observed sporadically in diverse groups of phages (79). Accessory genes may be essential under some conditions or provide a fitness advantage to the phage or its host (in the case of a prophage) under only certain conditions. In some cases, accessory genes have been referred to as morons; this term may be used to specifically refer to accessory genes of phages (5, 80, 81), as core and accessory genes are also a feature of bacterial genomes. The specific combination of accessory genes in a given phage genome likely reflects its adaption to a specific host or niche, meaning that deletion of accessory genes often will not result in phenotypic change in standard laboratory growth conditions on a permissive host. Indeed, *acr* genes are conditionally essential; they can be deleted or disrupted without phenotypic consequence when a phage is infecting a bacterial host lacking a CRISPR-Cas system or CRISPR spacers targeting that specific phage.

The best-studied phage accessory genes increase the fitness of the bacterial host during lysogeny, participating in adaptive lysogenic conversion (82, 83). Historically studied for their role in bacterial pathogenesis, diphtheria toxin, cholera toxin, and Shiga toxin are famous examples of prophage-carried accessory genes that dramatically alter the behavior of their hosts. Other conditionally essential phage accessory genes are involved in interphage warfare, preventing superinfection by a competitor phage. A recently published paper from the Hatfull group (84) highlights the diverse roles that phage accessory genes play during interphage antagonism. The group discovered accessory gene alleles in a phage that had evolved to counter a prophage-encoded toxin-antitoxin defense system. This example of phage-host interactions stands out as an example of Red Queen selection dynamics, which predict counteradaptation as a requirement for survival in arms races such as these (85). Previous examples of these dynamics have been demonstrated in both phages and eukaryotic viruses (86, 87).

Anti-CRISPR genes are another clear example of Red Queen dynamics at play in the phage accessory genome. These anti-immunity genes were first discovered in the genomes of a group of highly syntenic Mu-like phages with only a few pockets of genomic diversity—their accessory gene loci (40, 88). Interestingly, accessory gene loci appear in conserved locations across the genomes of these Mu-like phages, despite the sequences of the genes in the accessory locus being distinct. The anti-CRISPR accessory locus exemplifies the grab-bag nature of these loci—many diverse inhibitor proteins are encoded at the same location in the phage genome (**Figure 1**). It is interesting to broadly consider these loci as functional modules themselves—are genes in other syntenic accessory loci also inhibiting the same bacterial process through different mechanisms in these phages? In striking similarity to the *P. aeruginosa* Mu-like phages, the *L. monocytogenes* phages carrying *acrIIA* genes have highly syntenic genomes with conserved functional modules interspersed with accessory gene pockets.

The striking diversity of *acr* genes across even closely related phages generates several questions—for example, where were these diverse genes acquired from, and how did they evolve? No known proto-anti-CRISPRs have been discovered, and the evolutionary path of these novel proteins is mysterious. Analyses of the primary anti-CRISPR amino acid sequences have not revealed recognizable domains or motifs, and likewise, structural characterization of AcrF1 and AcrF3 has provided little insight into the origins of these inhibitors (54–56).

WHY ARE ANTI-CRISPR GENES SO DIVERSE?

Many distinct *acr* genes have been identified thus far for type I-E, I-F, II-A, and II-C CRISPR-Cas systems. Although the evolutionary history of anti-CRISPRs may be currently enigmatic, *acr*

diversity presents an intriguing question: Why are there so many *acr* genes? We propose two non–mutually exclusive hypotheses to explain *acr* diversity:

1. Distinct *acr* genes confer niche-specific fitness advantages to their host phage.
2. Diversity among *acr* genes is a form of distributed anti-immunity.

Hypothesis 1

As in the case of other phage accessory genes, the specific assemblage of *acr* genes possessed by a given phage represents a snapshot of a unique set of fitness challenges experienced by that phage. As an obvious example, the combination of *acrE* and *acrF* genes found in *P. aeruginosa* phages implies that they have cycled through hosts with both type I-E and type I-F CRISPR-Cas systems. It is less clear, however, what specific fitness advantages might be associated with using one particular AcrF protein over another. One immune inhibitor may impact phage fitness differently than another one does. In addition, the same Acr on different types of mobile elements, such as plasmids or islands, may lead to different fitness outcomes. Nonlytic conjugative elements impose different selective pressures on their hosts and thus would experience a different set of fitness costs and benefits associated with Acr deployment.

Because Acr efficacy is dependent on host Cas protein sequences and expression levels, some Acr proteins may incompletely inhibit CRISPR-Cas immunity within a given host. Although counterintuitive, this could benefit a phage by maintaining a population of infection-susceptible hosts and reducing selection for the evolution of alternative forms of antiphage immunity such as phage receptor loss. Indeed, it has been shown that under the presence of high phage burden, surface modifications are favored over CRISPR-based immunity (89). Furthermore, we also imagine the potential for Acr proteins to synergize with each other when two Acr-encoding phages infect the same bacterial cell. By targeting different steps in CRISPR interference, infections with heterotypic *acr* genes could lead to more viral replication than homotypic infections, selecting for the maintenance of diverse *acr* genes in a viral population and facilitating accessory gene exchanges between closely related phages.

Hypothesis 2

Diverse *acr* genes limit evolution of anti-anti-CRISPR (anti-Acr) mechanisms. An important facet of CRISPR-Cas immune function is the paradigm of distributed immunity, which is selection for the coexistence of many, equally fit immune alleles in a population. This theory of CRISPR-Cas immunity was proposed first by the Whitaker group (90) and tested using modeling approaches and experimentally evolved microbial populations of *Streptococcus thermophilus*. The distributed CRISPR-Cas immunity hypothesis is that viral predators select for the maintenance of a diverse spacer repertoire distributed across a microbial population. It is simple for a virus to escape targeting of one CRISPR spacer: A single point mutation can fully disable CRISPR immunity (37). However, distribution of many targeting spacers across a microbial population prevents individual viral escaper genotypes from emerging. Likewise, no single spacer will dominate the CRISPR landscape because immunity functions on the level of microbial populations rather than individual microbial genotypes.

To test the importance of distributed immunity, *P. aeruginosa* Mu-like phage DMS3 was used to infect artificially assembled populations of *P. aeruginosa* with varying degrees of spacer diversity distributed across the bacterial population (91). They found that low-diversity populations of *P. aeruginosa* with 1, 6, or 12 spacer genotypes routinely selected for the emergence of escaper phages that had presumably accumulated point mutations across protospacer regions. In contrast,

high-diversity populations with 24 or 48 spacer genotypes drove the DMS3 phage to extinction. In the case of high-diversity populations, only Acr deployment could protect the phage from CRISPR-Cas immunity. We invoke a hypothesis of distributed anti-immunity to describe anti-CRISPR diversity. By maintaining a diverse repertoire of *acr* genes, viral populations limit the emergence of bacterial anti-Acr mechanisms, such as point mutations in the target Cas protein. We next consider potential mechanisms for the emergence of anti-Acr strategies.

PUTATIVE ANTI-ANTI-CRISPR MECHANISMS

Acquisition of New CRISPR Systems

CRISPR-Cas immune systems are diverse; there are six known types, which can be further subdivided into many subtypes (20, 92). One of the simplest mechanisms by which bacteria could evolve to overcome phages with subtype-specific *acr* genes is to accumulate multiple types of CRISPR-Cas systems (**Figure 3**). In order to survive, a phage would need to inhibit all those systems. There are many examples of bacteria that have accumulated multiple types of CRISPR-Cas systems. *S. thermophilus*, the first organism in which CRISPR-Cas activity was demonstrated, has three to four different CRISPR-Cas systems: two type II-A systems, a type III-A system, and

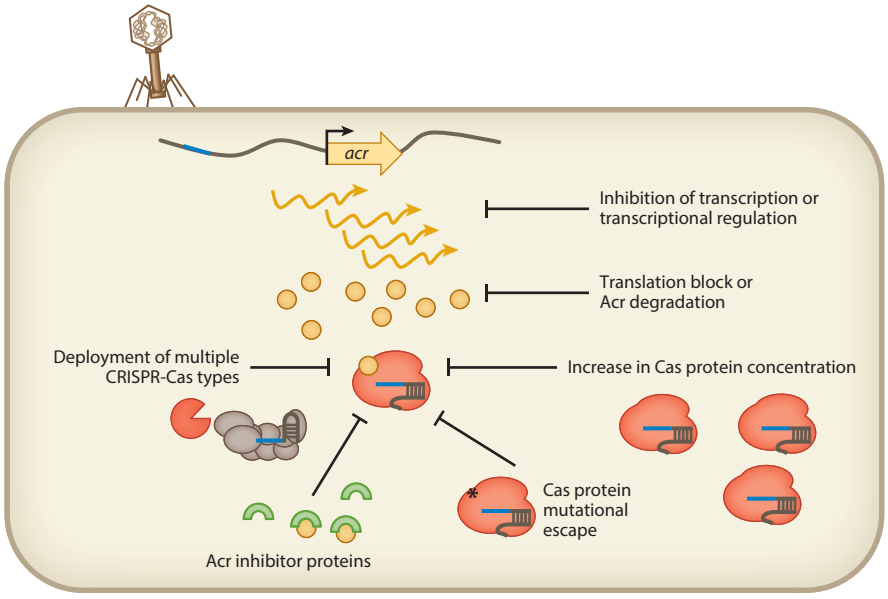


Figure 3

Characterized and predicted anti-anti-CRISPR mechanisms. To inhibit CRISPR-Cas immunity, *acr* genes need to be transcribed and translated inside a host cell. Currently, there are no described mechanisms by which bacterial hosts perturb anti-CRISPR transcript or protein levels, but AcrF proteins can lose efficacy when the intracellular concentration of Cas protein targets is increased. Cas mutations that lower or abolish Acr binding affinity for the Cas target could also serve to shift the balance in favor of the CRISPR-Cas system, as could protein inhibitors that sequester Acr proteins and prevent them from binding their Cas targets. Lastly, deployment of multiple types of CRISPR-Cas systems is a mechanism by which cells can protect themselves from subtype-specific Acr proteins and may in part explain the accumulation of multiple CRISPR-Cas system types and subtypes in diverse bacteria.

sometimes a type I-E system (93, 94). It is unknown whether *acr* genes have selected for this CRISPR-Cas diversity in *S. thermophilus*; however, it is notable that thus far *acr* genes inhibiting both type II-A and type I-E systems have been characterized. In contrast, no type III anti-CRISPR has been discovered. Similar to *S. thermophilus*, *Serratia* sp. ATCC39006 carries active type I-E, I-F, and III-A CRISPR systems, and these diverse systems were recently discovered to be regulated coordinately by quorum sensing (95). Again, it is unknown whether Acr proteins have driven selection for this *Serratia* species to carry multiple CRISPR-Cas systems; however, at least one *acrF* gene (*acrF8*) is found in *Serratia marcescens* genomes (49). Finally, in *P. aeruginosa*, acquisition of multiple CRISPR-Cas system subtypes may also be driven by CRISPR/Acr warfare. *P. aeruginosa* has both type I-F and type I-E CRISPR-Cas systems, which often co-occur in the same genome. Less frequently, *P. aeruginosa* genomes contain type I-C CRISPR-Cas systems that are mobilized on an integrative and conjugative element. Type I-C and type I-F also co-occur in *P. aeruginosa* genomes, but there are no examples yet of genomes carrying all three (43). Currently, no anti-CRISPRs against type I-C systems have been described.

Mutational Escape

The anti-CRISPRs that have been biochemically characterized bind specific surfaces on Cas proteins (53–55). By mutating these surfaces, bacteria could hypothetically evolve Acr-resistant CRISPR-Cas systems. By employing diverse inhibitors that bind to unique surfaces on CRISPR-Cas proteins, a population of viruses will limit accumulation of such CRISPR-Cas escape mutations (54). More information about the residue-specific interactions between Acr and Cas proteins will be critical to identify Acr-resistant CRISPR-Cas systems.

Regulatory Changes

The biochemically characterized anti-CRISPRs bind stoichiometrically to their Cas protein targets. Interestingly, overexpression of the type I-F CRISPR-Cas complex subunits in *P. aeruginosa* functions as an anti-Acr mechanism against the phages that use Acr proteins that bind this complex. In contrast, AcrF3, which targets the recruited Cas3 effector nuclease, is not affected by increasing the intracellular concentration of proteins that it does not bind (53). This shows that Acr proteins can be overwhelmed by shifting intracellular Cas protein concentrations, and suggests the possibility for bacteria to overcome anti-CRISPRs by overexpressing components of CRISPR-Cas systems. Multiple papers have reported different pathways involved in regulation of CRISPR-Cas systems in diverse bacteria (95–98). In each case, these systems are dynamically regulated, suggesting a cost to constitutive CRISPR expression. We hypothesize that Acr proteins that target CRISPR-Cas subunits more toxic to overexpress would have a selective advantage in this scenario. For instance, both Cas3 and Cas9 nucleases have the potential for genomic DNA cleavage, so Acr proteins that target these Cas proteins may be less susceptible to inhibition by CRISPR-Cas regulatory changes. Furthermore, some CRISPR-Cas systems are strongly induced during phage infection (99, 100). At first glance, this can be interpreted as enhanced immune activity, but this could also represent a mechanism to overwhelm inhibitor proteins deployed by the phage.

Dedicated Anti-CRISPR Inhibitors

Bacteria may possess dedicated inhibitors of Acr function, which prevent target binding or cause Acr protein degradation. Alternatively, blocking *acr* expression may also be possible; although

acr genes are themselves diverse in sequence and mechanism, they share the same regulatory environment. By targeting conserved, *cis*-acting DNA elements such as promoters, operators, and terminators required for *acr* expression, the bacterial cell could shift the balance in favor of the CRISPR-Cas system. For example, type I-E, I-F, and II-C anti-CRISPRs are associated with the conserved genes *aca1*, *aca2*, and *aca3* (of unknown function), whereas type II-A anti-CRISPR loci often carry *acrIIA1*. Though the functional relevance of these associated genes is currently unknown, they are strikingly conserved relative to their associated *acr* genes and could potentially represent the Achilles heel of an otherwise rapidly evolving system. A summary of these putative mechanisms for anti-CRISPR evasion is provided in **Figure 3**.

CRISPR MEETS ANTI-CRISPR IN LYSOGENY

Anti-CRISPRs are widespread across bacterial genomes. A recent report estimates that 64% of 449 *P. aeruginosa* type I-F CRISPR-Cas systems are inhibited by chromosomally encoded *acrF* genes (49). The same study concluded that the full diversity of type I-F systems across the phylum *Proteobacteria* could potentially be inhibited by known anti-CRISPRs. A separate analysis of *P. aeruginosa* type I-E systems estimated that 53% of 81 type I-E systems are inhibited by *acrE* genes (43). Similarly, >50% of type II-A systems in *L. monocytogenes* are estimated to be inhibited by the recently discovered *acrIIA* genes. Although CRISPR-Cas systems are commonly inhibited by Acr proteins, the consequences of CRISPR/Acr co-occurrence are relatively unexplored.

Self-Targeting

The stable coexistence of a CRISPR spacer and its target in the same cell is the most striking sign of inhibition of CRISPR-Cas activity. This scenario is commonly seen across the inhibited type I and II CRISPR-Cas systems mentioned above. In contrast, type III CRISPR-Cas systems, in which CRISPR activity is dependent on target transcription, have been demonstrated to conditionally tolerate their prophages with perfect protospacer matches (101).

CRISPR-Cas Alternative Functions

There is increasing evidence pointing toward CRISPR-Cas components (protein or RNA) performing alternative non-immunity-related functions (102). In the pathogen *Francisella novicida*, noncanonical activity of the CRISPR-Cas effector protein Cas9 in association with a small CRISPR-Cas-associated RNA (scaRNA) and the tracrRNA directly regulates levels of a virulence-associated transcript through base-paring with the RNA target (103). It is currently unknown whether *F. novicida* genetic parasites employ *acr* genes, but our current knowledge of type II inhibitors suggests the potential for undiscovered *acrIIB* genes to impact virulence regulation in *F. novicida*. Furthermore, a recent publication has shown RNA targeting in the *P. aeruginosa* type I-F CRISPR-Cas system, where a mismatched crRNA guides degradation of the *lasR* transcript, a master transcriptional regulator in *P. aeruginosa* (104). This noncanonical, RNA-directed CRISPR-Cas activity is dependent on both the type I-F CRISPR-Cas complex and the recruited effector nuclease Cas3. Do endogenous Acr proteins disrupt noncanonical CRISPR activity as well as canonical immunity functions? Given the widespread distribution of *acrF* genes in *P. aeruginosa* and beyond (49), *acr* genes have the potential to profoundly influence the biology of their bacterial hosts. It will be very interesting to see whether *acr* genes can inhibit, alter, or enable alternative functions, and what the evolutionary consequences of these interactions may be.

HORIZONTAL GENE TRANSFER

CRISPR-Cas immune systems, which destroy foreign DNA, can act as barriers to horizontal gene transfer. Whereas inhibition of viral parasites is an obvious adaptive function of a CRISPR-Cas system (27, 94), the exclusion of potentially beneficial foreign DNA, such as a prophage (83), can render a CRISPR-Cas system disadvantageous, and selection for CRISPR-Cas loss or inhibition can occur. By inhibiting CRISPR-Cas activity, chromosomal *acr* genes should enable foreign DNA acquisition in their hosts. Horizontal gene transfer is pervasive in bacteria and has had a profound impact on shaping bacterial genomes, suggesting a strong potential cost to CRISPR-Cas activity and a large potential benefit to anti-CRISPR acquisition.

Although individual examples of CRISPR-Cas systems excluding horizontal gene transfer mediated by plasmids and prophages and through natural transformation have been shown (105–108), it has been difficult to extrapolate these individual examples to broad principles of bacterial genome evolution. In 2015, the Koonin group (109) performed a bioinformatics study analyzing CRISPR-Cas activity (using CRISPR array length as a proxy for activity) and horizontal gene transfer across 1,399 microbial genomes. The authors found no evidence that CRISPR-Cas activity inhibited horizontal gene transfer on evolutionary timescales. Instead, they found that the best predictor of horizontal gene transfer was growth temperature, with lower genetic diversity occurring at hotter temperatures. This counterintuitive finding suggests that propensity for horizontal gene transfer is an intrinsic property of an organism and its ecological niche, and that CRISPR-Cas activity may exert its fitness impacts on the short-term population level rather than on long-term evolutionary timescales.

Emphasizing the population-level importance of CRISPR-Cas activity on horizontal gene transfer, a 2015 comparison of CRISPR-Cas distribution and horizontal gene transfer across a population of *P. aeruginosa* isolates demonstrated that CRISPR-Cas activity significantly restricted genome size (43). Importantly, each *P. aeruginosa* strain was only considered to be immune competent if it had a CRISPR array and *cas* genes and lacked chromosomal *acr* genes. The group showed that *P. aeruginosa* strains with active type I-E and I-F CRISPR-Cas systems had genomes that were on average 300 kbp smaller than those in *P. aeruginosa* strains with no CRISPR-Cas system. Fascinatingly, the authors also showed that *P. aeruginosa* strains with *acrE* or *acrF* genes had genome sizes that, on average, were not different from those of strains with no CRISPR-Cas system. Despite CRISPR-Cas inhibition likely being a relatively recent event in the evolutionary history of these bacterial strains, their horizontal gene transfer profile was similar to that of a strain that had presumably been without the CRISPR-Cas system for much longer. This result demonstrates the short-term, population-level impacts of CRISPR-Cas activity on bacterial genomes and emphasizes the rapid impacts that *acr* genes can have on the biology of their host bacteria.

The rapid acquisition of additional mobile genetic elements after CRISPR-Cas inhibition facilitates interactions among multiple mobile genetic elements. The coexistence of multiple mobile genetic elements in the same cell could increase the horizontal transfer of new genes, including *acr* genes themselves. It is interesting to consider the strong impacts that *acr* genes could have on shaping the accessory genomes of their host phage by "opening the door" to downstream infection. CRISPR-Cas inhibition of bacterial immunity could also have negative fitness impacts for the prophage, as the immune-compromised bacterial host could be infected and killed by a superinfecting competitor phage.

Not all phages have *acr* genes, suggesting that there may also be fitness costs to Acr action, such as licensing superinfecting phages. Interestingly, many of the Mu-like Acr phages in *P. aeruginosa* utilize diverse mechanisms to inhibit superinfection of other phages (40, 110), which

likely ameliorates some costs of host-immune compromise. As phage accessory genomes become better defined, it will be interesting to correlate the presence of superinfection exclusion genes with the presence of *acr* genes. Such complex genetic interactions in the phage accessory genome have likely profoundly shaped phage evolution, and may in part control anti-CRISPR distribution across phage populations.

CONCLUSION

CRISPR-Cas immune systems are a relatively recent discovery in the arms race between phages and their hosts, but they are likely ancient players in this battle. This new field has had a massive impact on our understanding of microbial evolution, phage biology, and horizontal gene transfer. Also remarkable is the elegance of many distinct, adaptive, sequence-specific RNA-guided nuclease systems possessed by bacteria, with some of them currently revolutionizing human gene editing and therapy. Anti-CRISPRs are an even more recent addition to the CRISPR story and are fascinating for many reasons, providing new insights into how CRISPR-Cas systems work, and how CRISPR-Cas systems and bacterial genomes have coevolved with the moving target of mobile DNA. While it is still early, we have already seen examples of both CRISPR-Cas systems and anti-CRISPRs shaping bacterial populations by dictating what DNA is horizontally acquired versus what is excluded. Furthermore, as CRISPR-Cas systems have revolutionized gene editing, anti-CRISPRs have provided new biotechnological resources in our efforts to precisely edit the human genome and develop new tools to probe it. Future work should focus on the discovery of new anti-CRISPRs that inhibit distinct CRISPR-Cas systems and on elucidation of their mechanisms of action. In addition, investigation of the counter-response from CRISPR-Cas systems to combat anti-CRISPR emergence will provide fascinating new insights into bacterial evolution.

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